

EXHALED AND PLASMA NITRITE: a comparative study among healthy, cirrhotic and liver transplant patients

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ABSTRACT - Context - There is a relative lack of studies about exhaled nitrite (NO_2^-) concentrations in cirrhotic and transplanted patients. **Objective** - Verify possible differences and correlations between the levels of NO_2^- , measured in plasma and exhaled breath condensate collected from patients with cirrhosis and liver transplant. **Method** - Sixty adult male patients, aged between 27 and 67 years, were subdivided into three groups: a control group comprised of 15 healthy volunteers, a cirrhosis group composed of 15 volunteers, and a transplant group comprised of 30 volunteers. The NO_2^- concentrations were measured by chemiluminescence. **Results** - 1) The analysis of plasma NO_2^- held among the three groups showed no statistical significance. 2) The comparison between cirrhotic and control groups, control and transplanted and cirrhotic and transplanted was not statistically significant. 3) The measurements performed on of NO_2^- exhaled breath condensate among the three groups showed no statistical difference. 4) When comparing the control group samples and cirrhotic, control and transplanted and cirrhotic and transplanted, there was no significant changes in the concentrations of NO_2^- . **Conclusion** - No correlations were found between plasma and exhaled NO_2^- , suggesting that the exhaled NO is more reflective of local respiratory NO release than the systemic circulation.

HEADINGS - Liver transplantation. Liver cirrhosis. Nitric oxide, diagnostic use.

INTRODUCTION

The increase in exhaled NO production has been reported in patients with advanced cirrhosis, where it is exhaled NO associated with systemic circulatory disorders⁽¹⁵⁾. Patients with liver cirrhosis often have several systemic hemodynamic disturbances, including hypotension, low systemic vascular resistance and reduced sensitivity to vasoconstrictors. With the progression of cirrhosis, continues to decrease vascular resistance, and low splanchnic blood pressure can lead to secondary disorders in kidney, liver blood flow and ascites. The precise mechanisms of these hemodynamic changes are not well understood. Excessive production of vasodilators such as prostacyclin, bradykinin, substance P and atrial natriuretic peptide, has been proposed, but there is no evidence that these are involved vasodilators⁽¹²⁾.

There is a relative lack of studies on liver disease changes in exhaled nitrite (NO_2^-) concentrations, a marker of injury, following liver transplantation. In this context, investigations on how exhaled NO concentrations vary in cirrhotic and transplanted patients will certainly contribute to new clinical findings. It is

worth mentioning that exhaled NO expresses micro-circulatory phenomena better than the measurement of NO_2^- levels in plasma samples collected from the pulmonary artery. Hence, assessment of exhaled NO may be more representative of vasoreactivity in the case of pulmonary hypertension. The initial hypothesis considered the possibility of hepatopulmonary syndrome (HPS), 33% of the population, which evolves with varying degrees of pulmonary hypertension caused by microvasculature disease, which makes the NO_2^- vented a possible biomarker being tested in patients with liver disease. Therefore, the aim of this study was to verify whether there are differences and correlations between the levels of NO_2^- , measured in plasma and exhaled breath condensate (EBC) collected from patients with cirrhosis and liver transplant.

METHOD

This study was approved by the Ethics Committee of the Hospital of the Faculty of Medicine of Ribeirão Preto of the University of São Paulo - FMRPUSP.

The study population included males between 27 and 67 years of age. The first group, control group

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(CG), was comprised of 15 healthy volunteers with a mean age 37.13 ± 8.95 years (13.333% ex-smokers). The second group, designated cirrhosis group (CiG), was composed of 15 cirrhotic subjects with a mean age of 53.40 ± 9.75 years (Table 1), and the third group (transplant group, or TxG) was composed of 30 subjects with a mean age of 54 ± 7.18 years (Table 2).

Fifteen healthy subjects without clinical criteria for cirrhosis (ascites, jaundice, and encephalopathy) were selected for the CG. The volunteers involved in the study were not current smokers, and they were not taking medications that could interfere with measurements of biomarkers of oxidative stress. The cirrhotic group was composed of 15 individuals from the liver transplant list, with clinical, laboratory, and liver biopsy findings suggestive of cirrhosis, with or without HPS. The transplanted group consisted of 30 individuals, who were at least 3 months status, post liver transplant performed at the FMRPUSP.

EBC and Blood samples were collected from a peripheral vein in the morning and transported on ice at -20°C . These samples were placed first in a bath set at 37°C and then centrifuged in a common centrifuge at 3,000 rpm for 10 minutes to obtain serum. All samples were stored in a freezer at -70°C for later dosing. The EBC collector device (Figure 1) was mounted in polystyrene box, and a mouth adaptor was added to the circuit, in order to aid patients in breathing in the tidal volume (TV). After correct placement of each circuit (mouth adaptor, plastic tubes, and Eppendorf/1.5 mL), the glass collector was positioned in the center of the polystyrene box which was filled with crushed ice. Care was taken so as not to allow direct contact between the two types of ice, and the measured temperature was around -10°C . Disinfection of devices and materials was accomplished with water and

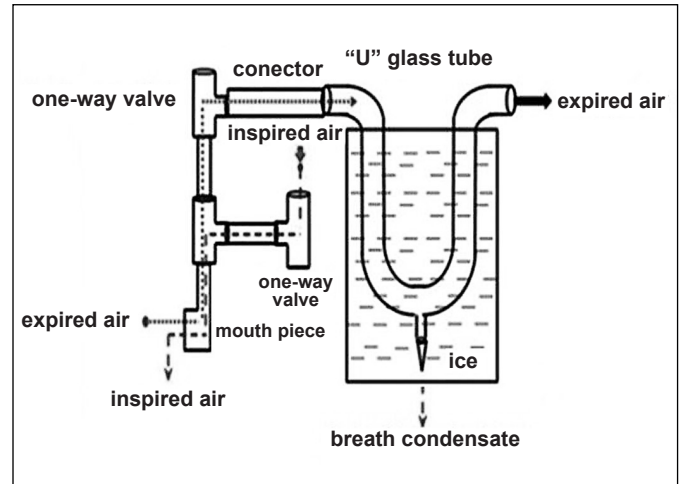


FIGURE 1. Scheme of the apparatus for collecting exhaled breath condensate (EBC)

neutral liquid detergent Extran MA O2 (Merck), followed by rinsing with tap water and Milli-Q water, and drying with medical grade compressed air. To minimize saliva contamination, room air breathing volunteers were asked to rinse their mouth prior the EBC collection. They were instructed to breathe in the TV through the piece adapted to the mouth by closing the lips around it. A calm respiratory pattern was requested during sampling, which lasted 10 minutes. For measurement of plasma EBC levels, 1.5 mL blood samples were collected through a peripheral venous access preoperatively, and through a central venous access postoperatively. The blood sample was placed in a standard collection tube (BD Vacutainer K2 EDTA) containing 0.08 mL heparin, transported on ice (-20°C), and centrifuged at 5,000 rpm for 10 minutes, at 4°C . The plasma was deproteinated and stored at -70°C for later NO_2^- level measurement. The plasma and EBC concentrations of NO_2^- were performed by chemiluminescence (Nitric Oxide Analyzer, 280i, brand-SIEVERS-Sievers NOA)^(2, 3, 13). The data were presented as mean \pm standard deviation. To compare groups of data dosages of plasma and EBC NO_2^- , one-way analysis of variance (one-way ANOVA) with Games-Howell post-test were used. Correlations were calculated by the Kendall's Tau-b test. Differences were considered statistically significant when $P < 0.05$. All analyses were performed with the aid of SPSS 15.0 (SPSS Inc, Chicago, IL, USA).

TABLE 1. Profile of the cirrhotic volunteers (cirrhosis group - CiG)

Cirrhosis Etiology	(%)	HPS (%)	Tabagism (%)
Alcohol	33.33	33.00	76.67 (non)
Álcohol/HCC	13.33		23.33 (ex)
Cryptogenic	20.00		
Virus C/Alcohol	6.67		
Virus C/ HCC	13.33		
Virus B/ HCC	6.67		
Virus B/Δ/ Álcool	6.67		

HPS: hepatopulmonary syndrome; HCC: hepatocellular carcinoma

TABLE 2. Profile of the liver transplanted volunteers (transplant group - TxG)

Cirrhosis Etiology	(%)	Immunosuppressive	(%)	Hepatitis B or C Treatment (%)	Tabagism (%)
Alcohol	40.00	Tacrolimus	36.67	13.33 Hepatite C	63.33 (non)
Cryptogenic	6.67	Cyclosporine	6.67	6.67 Hepatite B	36.66 (ex)
Virus C/Alcohol	6.67	Tacrolimus/Mycophenolate	23.33		
Virus C/ HCC	16.67	Cyclosporine/Mycophenolate	10.00		
Virus B	6.67	Sirolimus/Mycophenolate	20.00		
Virus C	26.67	Mycophenolate	3.33		

HCC: hepatocellular carcinoma

RESULTS

Differences were observed among the age of the control group (37.13 ± 8.95 years) and the age of cirrhotic (53.40 ± 9.75 years) and transplanted (54 ± 7.18 years). There was no difference in age between the groups of cirrhotic patients and transplanted.

The analysis of samples of plasma NO_2^- held among the three groups (CG = 9.02 ± 10.54 ; CiG = 3.97 ± 2.64 ; TxG = 4.92 ± 1.44), showed no statistical significance ($P = 0.561$). The comparison between cirrhotic and control groups ($P = 0.534$), control and transplanted ($P = 0.934$) and cirrhotic and transplanted ($P = 0.594$) was not statistically significant (Figure 2).

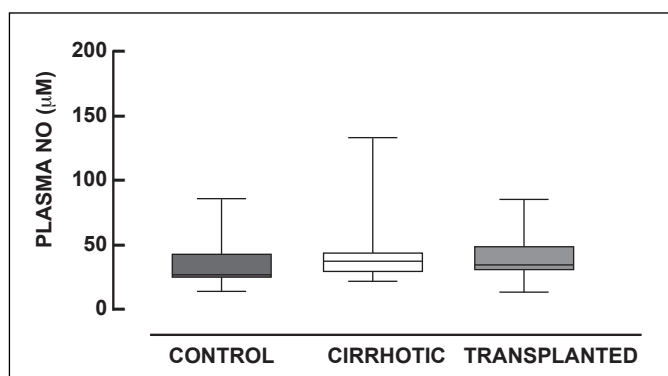


FIGURE 2. Dosage of plasma NO_2^- among the control ($n = 15$), cirrhotic ($n = 15$) and transplanted patients ($n = 15$). One-way analysis of variance (one-way ANOVA) with Games-Howell post-test were used. Data were considered statistically significant when $P < 0.05$

The measurements performed on samples of NO_2^- EBC among the three groups, control, cirrhotic and transplanted (CG = 36.97 ± 21.83 ; CiG = 47.95 ± 32.71 ; TxG = 39.18 ± 14.99) results showed no statistical difference ($P = 0.163$). However, there was a greater average value when analyzing NO_2^- control group samples. When comparing the control group samples and cirrhotic ($P = 0.202$), control and transplanted ($P = 0.321$) and cirrhotic and transplanted ($P = 0.411$), there was no significant changes in the concentrations of NO_2^- (Figure 3).

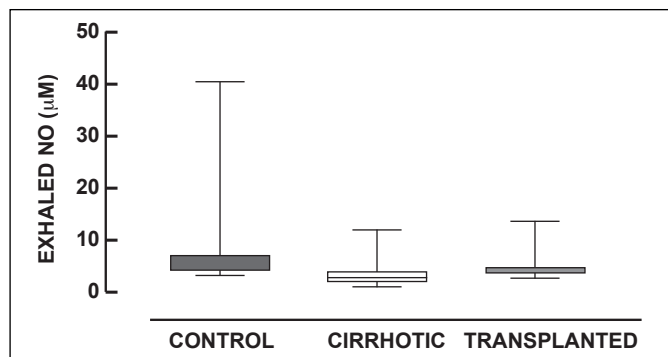


FIGURE 3. Dosage of exhaled EBC NO_2^- among the control ($n = 15$), cirrhotic ($n = 15$) and transplanted patients ($n = 15$). One-way analysis of variance (one-way ANOVA) with Games-Howell post-test were used. Data were considered statistically significant when $P < 0.05$

No correlations were found between plasma and exhaled NO_2^- calculated by the Kendall's Tau-b test.

DISCUSSION

In cirrhosis, decreased hepatic arterial and venous infusion may decrease of portal blood flow as a result of portal hypertension and blood flow may decrease due to impairment of autoregulation. However, patients with cirrhosis changes may be due to the systemic circulation and arteriovenous shunt splanchnic blood flow reduction. The reduction in hepatic perfusion becomes cirrhotic liver more susceptible to the effects of hypoxemia and hypotension during surgery⁽⁸⁾.

Patients with advanced liver disease have progressive pulmonary and splanchnic vasodilatation. This leads to a hyperdynamic circulatory state, which is associated with a high cardiac index (CI) and an increase of fluid in response to vasodilatation. Decreased arterial oxygenation, ranging from increased alveolar-arterial hypoxemia to severe, is commonly found in patients with cirrhosis. In the absence of obvious mechanical malfunction of the lungs, oxygenation abnormalities may be a consequence of the loss of ventilation-perfusion (V/Q), pulmonary shunt and/or diffusion of oxygen (O_2)⁽⁶⁾.

Vallance and Moncada (1991) hypothesized that NO may be the triggering factor of the hemodynamic changes in patients with liver cirrhosis. High concentrations of circulating endotoxin are often found in patients with cirrhosis who have no clinical evidence of infection⁽¹⁷⁾. So endotoxemia of cirrhosis can directly induce NOS in blood vessels or indirectly through cytokines, leading to increased synthesis and release of NO that may explain the hemodynamic abnormalities.

Sogni et al. showed that exhaled NO levels are increased in Child C cirrhotic patients compared with healthy subjects. The increase in exhaled NO and pulmonary vascular resistance decreased, suggest that increasing endogenous NO could explain the reduction in vascular tone severe cirrhosis⁽¹⁶⁾.

In the evaluation of plasma NO_2^- observed higher mean values for the cirrhotic group (47.95 ± 32.71) when compared with control groups (36.97 ± 21.83) and transplanted (39.18 ± 14.9). Results of the literature are controversial. High levels of NO_2^- and NO_3^- are found in patients with cirrhosis and chronic liver disease. Levels of NO_2^- and NO_3^- has been correlated with disease stage, considered as resulting from the hyperdynamic circulation often induced by portal hypertension. On the other hand, Becker et al. found a significant difference in the NO_2^- and NO_3^- concentrations of from transplant patients and healthy individuals during the phase prior anesthesia to the time of reperfusion⁽⁴⁾. Lluch et al. found that the plasma concentrations of NO were significantly higher in decompensated cirrhosis compared to compensated cirrhotic patients and control subjects, but no difference in the comparison between controls and compensated cirrhotic patients. In contrast, in this study, higher mean values of NO_2^- plasma were found to cirrhotic group compared to the control groups and transplanted⁽¹⁰⁾. The

differences between the studies published in the literature are probably associated with the clinical stages of the disease, number of patients enrolled, patients' age, the EBC collection methodology, and time of transplantation. In cases allocated in the present study it was observed an age markedly lower of the patients in the control group, the method of collecting the EBC was an original adaptation, cirrhotic patients were clinically stable, and the time of liver transplantation was more than one year.

The concentration of NO in exhaled air is decreased in patients after lung transplantation and in patients with acute respiratory distress syndrome (ARDS), but in relation to cardiac surgery, cirrhosis and liver transplant outcomes are being established recently^(1, 9, 11). In this study, the EBC dosages of NO₂⁻ among the three groups, control (10.54±9.02), cirrhotic (3.97±2.64) and transplanted (4.92±1.44) revealed no statistically significant results. Surprisingly, we observed a greater control group average value when analyzing the EBC NO₂⁻ samples. In addition, no increases were observed in the concentrations of NO₂⁻ in exhaled air of patients in the cirrhosis, or in the postoperative period of liver transplantation. Therefore, the exhaled NO₂⁻, in this sample of patients, there was not a reliable biomarker. Rolla et al. found that prior to liver transplant concentration of exhaled NO in cirrhotic patients was higher than in normal controls and after the procedure this concentration decreased and was significantly correlated with the decrease in alveolar-arterial gradient⁽¹⁴⁾. According to Matsumoto et al. the increase in exhaled NO is associated with systemic circulatory disorders in patients with liver cirrhosis⁽¹²⁾.

The correlation between plasma and exhaled NO seems unlikely, because of the NO short half-life the presence of oxygen, hemoglobin and reactive oxygen species. However,

it has been shown that NO may be preserved in vivo for binding to albumin, transported in this form and released elsewhere⁽⁷⁾. In the present study, there was no correlation between plasma NO₂⁻ and exhaled, regardless of the group examined. This finding is similar to results found by Biban et al. and Dillon et al., where no correlation between EBC and plasma NO levels were observed. This data may indicate that the exhaled NO, at least in normal conditions, are more reflective of local formation of NO than systemic^(5, 7).

Study limitation

Most suitable is the same patients were assessed before and after the transplant, thus avoiding the significant individual variations. The normal group (control) had an average age significantly different from the other groups, which could have influenced the outcome.

CONCLUSION

The NO₂⁻ measurements performed on samples of EBC among the three groups, control, cirrhotic and transplanted results showed no statistical difference. In addition, no correlations were found between plasma and exhaled NO₂⁻, suggesting that the exhaled NO is more reflective of local respiratory NO release than the systemic circulation.

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RESUMO - Contexto - Observa-se relativa falta de estudos sobre nitrito (NO₂⁻) exalado como biomarcador de lesão, após transplante de fígado. **Objetivo** - Verificar possíveis diferenças e correlações entre os níveis de nitrito (NO₂⁻), medido no plasma e condensado do exalado pulmonar de pacientes com cirrose e transplante de fígado. **Método** - Sessenta pacientes adultos, masculinos, idades entre 27 e 67 anos, foram divididos em três grupos: grupo controle composto por 15 voluntários saudáveis, grupo cirrose, composto por 15 voluntários e, grupo de transplante, composto por 30 voluntários. As dosagens plasmáticas e do condensado do exalado pulmonar foram realizadas por quimioluminescência. **Resultados** - A análise os valores de NO₂⁻ plasmático não mostrou diferença entre os grupos. As comparações entre grupos cirrose e controle, controle e transplantados e cirrose e transplante não foram significativas. As medidas em amostras de condensado do exalado pulmonar entre os três grupos não evidenciaram diferenças estatísticas. Ao comparar as amostras dos grupos controle e cirrótico, controle e transplantado e cirróticos e transplantados, não houve alterações significativas nas concentrações de NO₂⁻. **Conclusão** - Não foram encontradas correlações entre plasma e condensado do exalado pulmonar, sugerindo que o NO exalado reflete mais as condições respiratórias locais de liberação de NO do que a circulação sistêmica.

DESCRITORES - Transplante de fígado. Cirrose hepática. Óxido nítrico, uso diagnóstico.

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